View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Kidney International, Vol. 27 (1985), pp. 51–57

Effects of bilirubin on transepithelial transport of sodium, water, and urea

ANDREW S. BREM, WILLIAM J. CASHORE, MARYANN PACHOLSKI, JACQUELINE TETREAULT, and RONALD G. LAWLER

Department of Pediatrics, Rhode Island Hospital, and Division of Biology-Medicine, Department of Chemistry, Brown University, Providence, Rhode Island, USA

Effects of bilirubin on transepithelial transport of sodium, water, and urea. Urinary concentrating defects and renal salt wasting have been described in the hyperbilirubinemic Gunn strain of rat. Homozygous animals demonstrate significant reductions in renal medullary urea and sodium ion concentrations. These observations are consistent with possible bilirubin associated disorders in the transepithelial transport of water and solute. To test this hypothesis, measurements of active sodium transport and passive water and urea fluxes were made in hemibladders isolated from the Dominican toad, *Bufo marinus*. Tissues were exposed to amphibian bicarbonate Ringer's solution containing 0.1 mM bilirubin with 0.05% bovine serum albumin (BSA) or BSA alone. Vasopressin-stimulated sodium transport, as reflected by short circuit current (SCC), was inhibited by 18 ± 6% in the presence of bilirubin ($N = 10$; $P < 0.02$). Cyclic AMP (p-Cl-phenylthio cAMP 10^{-5} M) stimulated SCC was inhibited to a similar degree in the presence of bilirubin. The inhibition was noted only when bilirubin was in the serosal bath, and it could be abolished with BSA 0.5%. Bilirubin had no effect on the increase in SCC induced by higher concentrations of cyclic AMP (10^{-4} M), aldosterone, or amphotericin B. Furthermore, bilirubin had no effect on the hydro-osmotic response to vasopressin and vasopressin-induced changes in urea permeability. These findings show that short-term exposure to bilirubin exerts a tissue-specific effect on the vasopressin-stimulated active transport of sodium but has no effect on the vasopressin-induced fluxes of water and urea.

Effets de la bilirubine sur le transport transépithélial de sodium, d'eau, et d'urée. Des défauts de concentration urinaire et une fuite rénale de sodium ont été observés dans la souche de rats hyperbilirubinémiques Gunn. Les animaux homozygotes présentent une réduction significative des concentrations en urée et en ion sodium dans la médullaire rénale. Ces observations sont compatibles avec des désordres peut-être liés à la bilirubine du transport transépithélial d'eau et de solutés. Afin de tester cette hypothèse, des mesures du transport actif du sodium et des flux passifs d'eau et d'urée ont été effectuées dans des hémi-vessies isolées à partir du Crapaud Dominicain *Bufo marinus*. Les tissus étaient exposés à une solution de Ringer bicarbonate pour amphibiens contenant 0,1 mM de bilirubine avec 0,05% de sérum albumine de boeuf (BSA) ou de la BSA seule. Le transport de sodium stimulé par la vasopressine reflété par le courant de court circuit (SCC) était inhibé de 18 ± 6% en présence de bilirubine ($N = 10$; $P < 0,02$). Le SCC stimulé par l'AMP cyclique (p-Cl-phénylthio cAMP 10^{-5} M) était inhibé de façon semblable en présence de bilirubine. L'inhibition était notée uniquement quand la bilirubine était dans le bain séreux, et ne pouvait être abolie avec 0,5% de BSA. La bilirubine n'avait pas d'effet sur l'augmentation de SCC induite par des concentrations plus élevées de cAMP (10^{-4} M), par l'aldostérone ou par l'amphotéricine B. De plus, la bilirubine était sans effet sur la réponse hydro-osmotique à la vasopressine, ni sur les modifications de la perméabilité à l'urée induite par la vasopressine. Ces résultats montrent qu'une courte exposition à la bilirubine exerce un effet spécifique de tissu sur le transport actif du sodium stimulé par la vasopressine, mais est sans action sur les flux induits par la vasopressine d'eau et d'urée.

While the effects of hyperbilirubinemia on the central nervous system remain paramount, the toxicity of this pigment has been clearly demonstrated in other organs including the kidney [1–4]. The Gunn strain of rat with congenital absence of hepatic glucuronyl transferase and chronic unconjugated hyperbilirubinemia develops significant polyuria. When studied in greater detail, these animals uniformly show a limited free water clearance, increased fractional sodium excretion, and deposits of crystalline bilirubin in the papilla of the kidney [1–3]. In addition to its renal effects, high concentrations of unconjugated bilirubin promote the secretion of water and sodium in the isolated perfused intestine of the hamster [4]. Taken together, these data are consistent with the view that bilirubin may alter the epithelial transport of solute and water.

Although the data gathered in the Gunn rat are highly suggestive of altered renal epithelial transport, the effects of bilirubin are less clear in the human infant. Broberger and Aperia [5] demonstrated that hyperbilirubinemic newborns given a salt and water load had a decreased glomerular filtration rate (GFR) and a higher fractional sodium excretion than nonhyperbilirubinemic control infants. However, Engle and Arant [6] performed a similar clinical study on hyperbilirubinemic newborns and failed to show any significant differences in GFR or in the tubular handling of sodium. These infants were not challenged either with a salt or water load. If high concentrations of bilirubin change renal function in the newborn infant, the alterations may be subtle and may depend on a more prolonged exposure.

The urinary concentrating and diluting capabilities of the kidney depend on the epithelial transport of solute and water. Because evidence indicates that bilirubin may interfere with this epithelial cell function, the present study was designed to examine the effects of unconjugated bilirubin on transepithelial transport of sodium, water, and urea. The toad urinary bladder was chosen as the experimental model, for it is essentially a single epithelial cell membrane which, in many respects, mimics the distal nephron of the mammalian kidney. Under the influence of vasopressin, it actively transports sodium and

Received for publication March 14, 1984,
and in revised form July 31, 1984

© 1985 by the International Society of Nephrology

allows for the passive reabsorption of urea and water [7]. Data gathered from these studies show that stimulated sodium transport may be affected by unconjugated bilirubin. The inhibition of vasopressin-stimulated sodium transport by bilirubin was seen in preparations derived from previously well-hydrated animals in whom a salt-losing tendency would not be expected.

Methods

Dominican toads (*Bufo marinus*) were obtained from National Reagents, Bridgeport, Connecticut, USA. All toads were sacrificed by double pithing. Bladders were then removed and used for selected experiments as outlined below. The bladders were bathed in an amphibian Ringer's solution (sodium 114 mEq/liter, potassium 3.2 mEq/liter, bicarbonate 2.4 mM, chloride 113.4 mEq/liter, calcium 0.89 mM) which has an osmolality of 218 mOsm/kg water and a pH of 8.1. A concentration of 0.1 mM bilirubin (~5 mg/dl) was used for all experiments. The bilirubin was initially dissolved in 0.1 N NaOH and then added to the amphibian Ringer's solution, which was found to be the maximum concentration of bilirubin that would consistently stay in solution at this alkaline pH. Unless otherwise stated, albumin 0.05% was present in both control and bilirubin-containing solutions. The albumin had no effect on sodium transport, water movement, or urea transport in preliminary investigations, and it served to maintain the bilirubin's solubility in an otherwise supersaturated solution.

The cyclic AMP salt used was 8 (p-chlorophenylthio)-adenosine 3′5′ cyclic phosphate, monosodium dihydrate, ICN Pharmaceuticals, Irvine, California, USA. Vasopressin was obtained from Parke, Davis and Company, Detroit, Michigan, USA. Isotopes were from New England Nuclear Corporation, Boston, Massachusetts, USA. Aldosterone was from Steraloids, Inc., Wilton, New Hampshire, USA. Bilirubin and bovine serum albumin (BSA) were obtained from Sigma Chemical, St. Louis, Missouri, USA. Amphotericin B was from ER Squibb, Inc, Princeton, New Jersey, USA.

Because the toad urinary bladder is bilobed, hemibladders from the same animal were used in control and experimental preparations. Thus, all observations were paired and analyzed statistically using Student's paired *t* test. Results were expressed as the mean ± the SE.

Determination of sodium transport

The measurement of sodium transport across the toad bladder was determined by the short circuit current (SCC) technique of Ussing and Zerahn [8]. Hemibladders were mounted onto a lucite chamber (Nicholson Precision Instruments, Gaithersburg, Maryland, USA). Both surfaces of the hemibladder were bathed in the amphibian Ringer's solution bubbled with compressed air. After 60 min of equilibration, both serosal and mucosal bathing solutions were changed. Unless specifically mentioned, the mucosal bath consisted of amphibian Ringer's solution while the serosal bath contained Ringer's with albumin (control) or albumin and bilirubin (experimental). The lucite chambers were covered to prevent light exposure. Tissues were then bathed in these solutions for 45 min prior to the addition of any stimulatory test substance. Short circuit current was recorded throughout the equilibration period, the bilirubin exposure period, and the stimulation period. Transepithelial potential difference (PD) was only recorded during the equilibration and bilirubin exposure periods.

In the initial series of experiments, the tissues were stimulated with vasopressin (40 mU/ml) added to the serosal bath. Since the peak response in SCC occurred within 30 min and was followed by a rapid return toward baseline, the vasopressin stimulation period was limited to 60 min. This procedure was repeated with a number of variations which included increasing the albumin in the serosal bath to 0.5% in control and experimental situations, placing the bilirubin-albumin solutions on the mucosal rather than the serosal side of the bladder, and substituting cyclic AMP (p-chlorophenylthio cAMP) 10^{-5} or 10^{-4} M for vasopressin.

Aldosterone (10^{-7} M) was studied in the next series of SCC experiments. To suppress endogenous aldosterone production, toads were soaked in 0.6% saline for at least 48 hr prior to sacrifice. Bladders were excised, mounted onto the chambers, and left overnight bathed in a Ringer's solution containing penicillin G 0.1 mg/ml, streptomycin 0.1 mg/ml, and glucose 10 mM to further deplete them of endogenous hormone. On the morning of the experiment, the serosal and mucosal bathing solutions were replaced with Ringer's solution which did not contain antibiotics or glucose. Bladders were short-circuited to test viability. Bathing solutions were again changed so that the experimental tissues were exposed to bilirubin. Aldosterone was then added to the serosal medium of both experimental and control hemibladders and readings of SCC were made for 360 additional minutes.

In the third series of experiments, SCC was stimulated by the antifungal agent amphotericin B (2.5 μg/ml) placed in the mucosal bath of both experimental and control tissues. Bladders were prepared as described in the initial vasopressin experiments. SCC measurements were recorded for 90 min following the addition of amphotericin B to the mucosal solutions.

Considerable variation in the absolute value of SCC exists between hemibladders. To standardize the experimental data for comparison, SCC was expressed as a ratio reflecting the change rather than the absolute value. This ratio was derived by dividing the measured SCC at given time t by the measured SCC obtained prior to the addition of any drug, t_0. Experimental hemibladders were compared to matched controls using the equation shown below:

$$\frac{\dfrac{SCC_t}{SCC_{t_0}}\ \text{Exp}}{\dfrac{SCC_t}{SCC_{t_0}}\ \text{Control}} \qquad (1)$$

Measurement of osmotic water movement

Osmotic water flow across the isolated toad urinary bladder was determined gravimetrically using the Bentley bag technique [9]. While electrical parameters were not measured, tissue integrity was indirectly assessed by membrane impermeability in the absence of hormone and maximal water flow in the presence of hormone. The paired hemibladders were dissected out and each hemibladder was tied to a short glass tube forming a bag. The hemibladder bags were rinsed twice and then filled with 5 ml of deionized water. Each bladder was suspended in 50 ml of amphibian Ringer's solution containing 0.05% albumin.

The experimental Ringer's solution also contained bilirubin. Hemibladders were removed from the bath, gently blotted on filter paper, and weighed on a balance (Mettler H-35 AR, Mettler Instrument Corporation, Hightstown, New Jersey, USA) to determine weight loss as a measure of water flow. After a control period of 45 min, bladders were exposed to vasopressin 20 mU/ml and weights were measured at 15-min intervals for 60 min. In selected experiments, the hydro-osmotic response was induced with vasopressin 1 mU/ml or cyclic AMP placed in the serosal bathing medium in a concentration of 10^{-5} M. Again, all experiments were performed in a darkened room with the individual beakers wrapped with aluminum foil to minimize exposure to light.

Measurement of urea permeability

Urea permeability was assessed by the method of Maffly et al [10]. Hemibladders were mounted onto lucite chambers and short-circuited in a similar fashion to that described in the SCC studies. To prevent bacterial breakdown of urea, the Ringer's solution contained penicillin G, 0.1 mg/ml, and streptomycin, 0.1 mg/ml. The presence of antibiotics in the bathing solution had no demonstrable effect on either basal or vasopressin-stimulated urea fluxes (basal with antibiotics 16.5 ± 4.2 × 10^{-7} cm^2 sec, basal without antibiotics 16.8 ± 4.9 × 10^{-7} cm^2 sec, $N = 4$; and VP stimulated with antibiotics 109 ± 8.4 × 10^{-7} cm^2 sec, VP stimulated without antibiotics 105 ± 1.4 × 10^{-7} cm^2 sec, $N = 8$). ^{14}C-labeled urea was added to Ringer's containing 2 mM cold urea bathing the mucosal surface of the hemibladders. Samples of the serosal and mucosal bath were then taken for counting every 30 min for a 1-hr control period. Vasopressin, 40 or 1 mU/ml, was then added to the serosal bathing medium. Samples of both mucosal and serosal bathing solutions were taken for counting every 15 min following vasopressin exposure for a total of 1 hr. The samples were then counted in a liquid scintillation spectrometer (Searle Analytical Mark III, Chicago, Illinois, USA) for 10 min to achieve a counting rate 1,000 times above background. In initial experiments, Ringer's containing bilirubin was present on the serosal side with mucosal to serosal urea flux observed. Similar urea fluxes were measured with bilirubin in the mucosal bath. Lastly, the back flux of urea (serosal to mucosal) was determined with both bilirubin and urea present in the serosal medium. As previously mentioned, the chambers were covered to prevent breakdown of bilirubin caused by light exposure. The permeability coefficient, K_{trans}, for urea was then calculated according to the formula below:

$$K_{trans} = \frac{\text{increase in counts on the unlabeled side}}{\text{number of counts on the labeled side} \times \text{membrane area} \times \text{time}} \quad (2)$$

$$\text{Membrane area} = 3.06\ cm^2$$

^{31}P NMR analysis

^{31}P nuclear magnetic resonance analysis of the tissues was undertaken to assess qualitative changes in intracellular high energy phosphate compounds in the presence and absence of bilirubin. Hemibladders from three toads were incubated in amphibian Ringer's containing 0.05% BSA and bilirubin 0.1 mM for 45 min. Vasopressin, 40 mU/ml, was then added to the bath and the incubation was continued for an additional 30 min. The tissues were then transferred into a 10 mm NMR tube cooled to 4°C containing Ringer's with 0.05% BSA, bilirubin, and vasopressin. NMR spectra were obtained on the sample over the next 30 min at 4°C following the method of Bond et al [11]. The procedure was repeated with the matched control hemibladders; bilirubin was absent from the bath.

The scans were generated using a Fournier transform nuclear magnetic resonance spectrometer (Bruker WM 250 Bruker Instruments, Billerica, Massachusetts, USA) with an operating frequency of 101.3 MHz for ^{31}P. A 90° pulse with a recycle time of 1.5 sec was used in these studies. The Ringer's in the NMR tubes was made up with 20% D_2O for a field-frequency lock. Spectra were processed with a line broadening of 50 Hz.

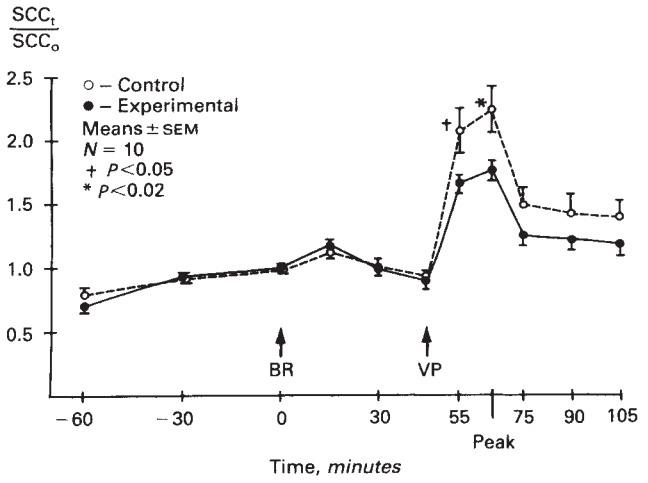

Fig. 1. *Effect of bilirubin on vasopressin-stimulated short circuit current (SCC).* While bilirubin 0.1 mM had no effect on basal SCC (control 58.1 ± 6.0 μAmps vs. BR 49.3 ± 7.5 μAmps), the peak response to vasopressin was inhibited by 18 ± 6% (control-VP 143.9 ± 15.3 μAmps vs. BR-VP 99.3 ± 14.1 μAmps) ($N = 10$; $P < 0.02$).

Results

Effect of bilirubin on sodium transport

Bilirubin in the serosal bathing medium appeared to have no effect on basal SCC and PD during a 45-min observation period. With vasopressin stimulation, the peak SCC response in bilirubin-exposed bladders was 18 ± 6% less than in matched controls ($P < 0.02$; $N = 10$; Fig. 1). When this experiment was repeated with 0.5% rather than 0.05% BSA and bilirubin in the serosal medium, the inhibitory effects of bilirubin on SCC disappeared. Moreover, bilirubin and 0.05% BSA in the mucosal bath had no effect on basal or vasopressin-stimulated SCC.

Since vasopressin action is mediated in the epithelial cell through the generation of cyclic AMP, the effect of bilirubin on cyclic AMP-stimulated sodium transport was next examined. As before, bilirubin in the serosal medium did not affect basal SCC or PD. Following stimulation with the cyclic AMP analog, 10^{-5} M, the peak rise in SCC was inhibited by 24 ± 5% ($P < 0.01$; $N = 11$; Fig. 2), a response similar to that seen earlier with

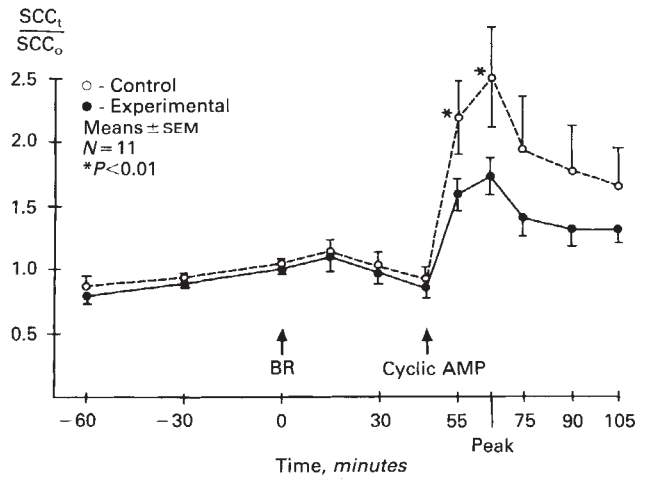

Fig. 2. *Effect of bilirubin on cyclic AMP-stimulated short circuit current (SCC).* Basal SCC was unaffected by bilirubin (control 49.5 ± 9.7 μAmps vs. BR 52.2 ± 7.1 μAmps). Bilirubin inhibited the peak response to cyclic AMP (10^{-5} M) by 24 ± 5% (control cyclic AMP 120.6 ± 18.8 μAmps vs. BR-cAMP 99.5 ± 11.1 μAmps) ($N = 11$; $P < 0.01$). The inhibition could be abolished with cyclic AMP (10^{-4} M).

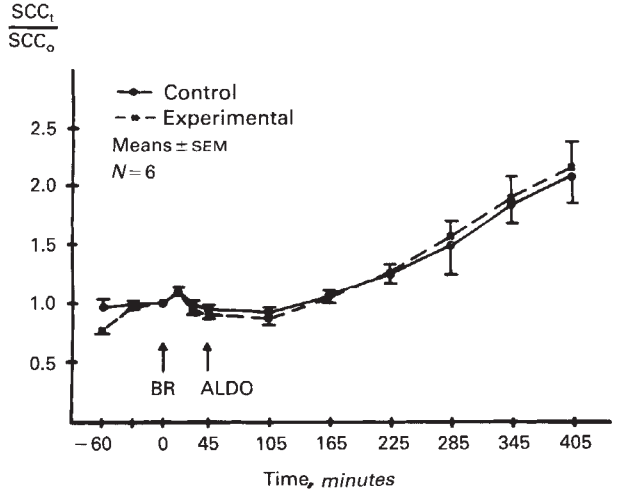

Fig. 3. *Effect of bilirubin on aldosterone-stimulated short circuit current (SCC).* The aldosterone-stimulated increase in SCC was unaffected by bilirubin over the entire 360 min of the study.

the vasopressin. A supramaximal dose of cyclic AMP, 10^{-4} M, overcame the effects of bilirubin.

Aldosterone also stimulates sodium transport in the toad urinary bladder, but the transport depends on protein synthesis [12]. Thirty to ninety minutes may elapse before an increase in SCC is observed. After a 45-min exposure to bilirubin, aldosterone was added to both experimental and control bladders. In six pairs of experiments, bilirubin appeared to have no influence on aldosterone-stimulated SCC over the 360-min observation period (Fig. 3).

The effect of bilirubin on the sodium pump was indirectly tested when the mucosal surfaces of the hemibladders were incubated with the standard amphibian Ringer's solution containing amphotericin B (2.5 μ/ml). Amphotericin B, an antifungal agent, alters the mucosal barrier to sodium entry resulting in a transient increase in sodium transport, reflected

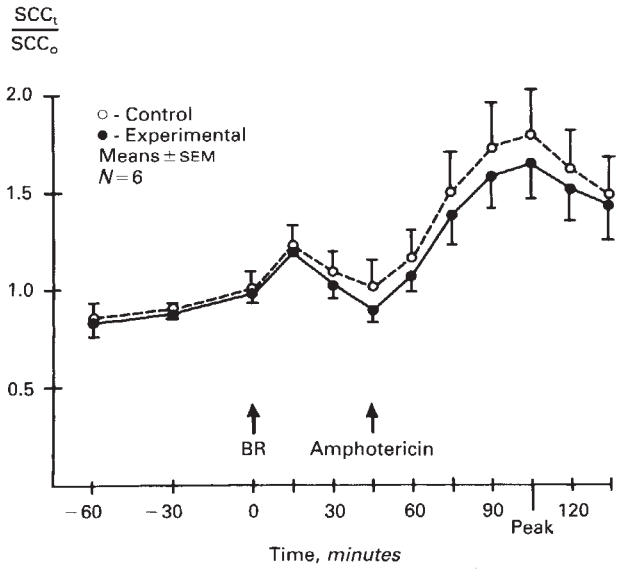

Fig. 4. *Effect of bilirubin on amphotericin B-stimulated short circuit current (SCC).* Bilirubin in the serosal bath did not influence the increase in SCC produced by amphotericin B (2.5 μg/ml) in the mucosal bath.

by a rise in SCC [13]. As before, basal SCC was unaffected by bilirubin in the serosal bath. Incubation with amphotericin B caused a similar increase in both control and bilirubin-exposed bladders ($N = 6$; Fig. 4).

Effect of bilirubin on the hydro-osmotic response to vasopressin and cyclic AMP

A 30-min incubation with bilirubin in the serosal bath had no effect on basal water movement. When vasopressin (20 mU/ml) was added to the serosal bath, no significant difference in the hydro-osmotic response to the hormone was noted. Mean water flow for control tissues was 46.5 ± 2.7 mg/min, while experimental bladders demonstrated water flow of 45.5 ± 3.3 mg/min ($N = 9$). Similar results were seen with vasopressin 1 mU/ml; control 41.0 ± 5.3 mg/min versus experimental 44.9 mg/min, $N = 6$. When tissues were stimulated with the cyclic AMP analog at 10^{-5} M, the hydro-osmotic response was again not affected by the presence of bilirubin in the serosal bathing medium. Peak water flow in control tissue averaged 24.2 ± 3.5 mg/min, while experimental tissues demonstrated water flow of 21.0 ± 2.9 mg/min ($N = 6$).

Effect of bilirubin on urea permeability

Bilirubin in the serosal bathing medium did not appear to influence the basal mucosal to serosal flux of urea as reflected by the urea permeability coefficient, K_{trans}. When the bladders were stimulated with vasopressin, 1 or 40 mU/ml, K_{trans} for urea increased tenfold in both control and experimental tissues and was unaffected by the bilirubin. When the experiment was repeated with bilirubin and urea present in the mucosal bathing medium, no statistical difference in basal or vasopressin-stimulated K_{trans} was seen (Table 1). Lastly, bilirubin in the serosal bath did not influence vasopressin (40 mU/ml) stimulated serosal to mucosal backflux of urea (K_{trans} control 62.3 ± 6.1 ×

Table 1. Urea permeability (K_{trans}) in the presence of bilirubin (0.1 mM)[a]

Group	Urea $K_{trans} \times 10^{-7}$ cm²/sec ± SEM							
	Basal	*N*	Vasopressin *40 mU/ml*	*N*	Basal	*N*	Vasopressin *1 mU/ml*	*N*
Bilirubin serosal bath								
Control	6.7 ± 1.8	8	76.3 ± 4.6	12	3.0 ± 0.7	8	78.0 ± 9.2	16
Experimental	6.6 ± 2.0	8	72.2 ± 4.2	12	3.9 ± 0.7	8	75.0 ± 8.9	16
Bilirubin mucosal bath								
Control	2.6 ± 0.3	7	70.4 ± 5.1	10	2.3 ± 0.3	5	71.0 ± 7.2	16
Experimental	2.2 ± 0.4	7	65.8 ± 5.2	10	2.3 ± 0.8	5	68.0 ± 6.7	16

[a] Mucosal-to-serosal fluxes of ^{14}C urea were measured in the basal and vasopressin-stimulated state (40 and 1 mU/ml).

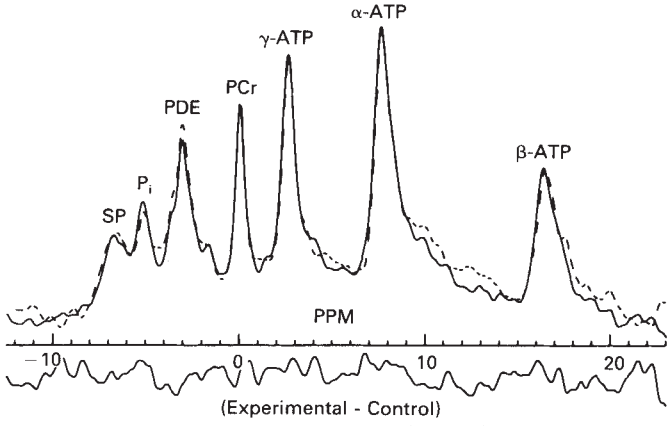

Fig. 5. *^{31}P NMR analysis.* The presence of bilirubin had no demonstrable effect on any of the high energy phosphate peaks. When control spectra (*dashed line*) were subtracted from experimental spectra (*solid line*) a signal indistinguishable from background noise resulted. Each peak is labeled: SP, sugar phosphates; P_i, inorganic phosphate; PDE, phosphodiesters; P_{Cr}, creatine phosphate; γATP, γATP + βADP; αATP, αATP + αADP; and βATP = βATP.

10^{-7} cm²/sec vs. K_{trans} experimental 62.5 ± 8.1 × 10^{-7} cm²/sec; $N = 6$).

^{31}P NMR analysis

Spectra from two separate experiments averaging 1200 scans each were pooled for both experimental and control tissues depicted in Figure 5. No significant differences in the high energy phosphate peaks were observed in hemibladders exposed to bilirubin as compared to controls. Subtraction of the pooled experimental spectra from the pooled control spectra revealed a signal indistinguishable from background noise. The creatine phosphate to γ ATP ratio in both sets of spectra was 0.5, a ratio similar to 0.6 ± 0.1 reported by Bond et al [11] in the toad urinary bladder. The times of tissue exposure to bilirubin and bilirubin with vasopressin paralleled the previously described incubation periods when bilirubin inhibited vasopressin-stimulated SCC.

Discussion

Bilirubin nephropathy in homozygous Gunn rats has been characterized by urinary concentrating defects, increased sodium wasting, and a decreased cortical medullary sodium concentration gradient, all associated with crystalline bilirubin in the renal medulla [1–3]. Gunn weanling rats demonstrate many of these changes by 21 days of age [3]. Moreover, these animals have lower concentrations of urea and sodium in the renal medulla compared to control heterozygous or light-treated homozygous litter mates [3]. From these and other observations, Martinez-Maldonado, Suki, and Schenker [2] suggested that the defects in urinary concentrating ability are directly related to unconjugated bilirubin impairing solute and/or water transport in the loop of Henle. Sodium and urea are the major solutes which account for the maintenance of renal medullary hypertonicity [14]. Thus, bilirubin-related diminished transport of either or both of these solutes could possibly influence the ability of the kidney to concentrate or dilute urine. In addition, the hydro-osmotic response to vasopressin in renal collecting ducts may be directly influenced by bilirubin. The present studies, conducted with the toad urinary bladder, examined the effect of bilirubin on the transepithelial transport of these solutes and water.

Short-term exposure to bilirubin in the concentration tested appeared to have no direct effect on basal SCC or PD. Bilirubin in the serosal bath resulted in a significant inhibition in vasopressin-stimulated SCC. The inhibitory effects of bilirubin were observed only with bilirubin on the serosal or blood side of the epithelial membrane and could be eliminated by increasing the albumin in the serosal bath to 0.5%. This is consistent with the increased binding of bilirubin to albumin, thereby preventing bilirubin interaction with the epithelial cell.

Vasopressin action is mediated in the cell by cyclic AMP through stimulation of adenylate cyclase, an enzyme located at or near the serosal membrane [15]. The inhibitory effect of bilirubin on vasopressin-stimulated SCC could be related in part to an alteration in the vasopressin membrane receptor or an effect on the enzyme, adenylate cyclase. Bilirubin has been shown to inhibit adenylate cyclase isolated from fat cells [16]. In our experiments, bilirubin also inhibited cyclic AMP (10^{-5} M) stimulated SCC. Thus, alterations proximal to the generation of cyclic AMP, for example, in the membrane receptor or in adenylate cyclase, seem to be unlikely explanations for the diminished SCC response. The observed effects of bilirubin on vasopressin and cyclic AMP (10^{-5} M) stimulated SCC would be consistent with a selective bilirubin-induced inhibition of a specific cyclic AMP-dependent protein kinase. Diminished cyclic AMP-dependent protein kinase activity has been described in rabbit brain, and the bilirubin-induced change in enzyme activity was partially or completely restored with the phosphodiesterase inhibitor, aminophylline [17]. Like

aminophylline, the higher concentration of cyclic AMP (10^{-4} M) in our experiments may have overcome the effect of bilirubin on this enzyme.

Aldosterone also stimulates sodium transport in the toad urinary bladder, but it does so by a mechanism independent of cyclic AMP generation [12]. Bilirubin appeared to have no major effect on the entry of aldosterone into the cells or on the intracellular apparatus associated with sodium transport. Amphotericin B indirectly tests the integrity of the serosal membrane sodium pump [13]. Here, as with aldosterone, bilirubin (0.1 mM) had no effect on the increased Na-K ATPase pump activity induced by the rise in cytosolic sodium. Although Na-K ATPase isolated from human red blood cells [18] and rat brain [19] can be inhibited by similar concentrations of bilirubin, the effect is temperature-dependent, becoming apparent when the temperature falls below 20°C. Since the present experiments were conducted at 25°C, the expected inhibition of NA-K ATPase would be minimal, beyond the sensitivity of this bioassay.

Collectively taken, these data lead one to suggest that the serosal sodium exit step is not the site of inhibition to vasopressin-stimulated sodium transport. Because bilirubin did not inhibit aldosterone, cyclic AMP at 10^{-4} M, or amphotericin B-stimulated sodium transport, it seems unlikely that the inhibitory effects on vasopressin-stimulated SCC are the result of a nonspecific alteration in the oxidative metabolism necessary for active sodium transport [12]. Although it represents a qualitative analysis, the ^{31}P NMR spectra, obtained when the inhibition of vasopressin-stimulated SCC was known to occur, demonstrated no alteration in the ATP or Pi peaks in the presence of bilirubin. This observation would also go against a generalized suppression of oxidation metabolism and an inhibition of Na-K ATPase due to decreased availability of ATP. The inhibitory effect of bilirubin on vasopressin-stimulated SCC appears to be at a site following the generation of cyclic AMP and could well involve vasopressin-induced changes in mucosal membrane sodium conductance.

In contrast to the SCC studies, the hydro-osmotic response to vasopressin and cyclic AMP was unaffected by the presence of bilirubin in the serosal bath. Vasopressin interacts with its specific serosal membrane receptor, activating adenylate cyclase to produce cyclic AMP. A cascade of intracellular events then occur which result in alterations in the apical or mucosal membrane of the epithelial cell and allow for passive transmembrane water movement along an osmotic gradient [15]. The fact that bilirubin does not interfere with this process further indicates that the vasopressin membrane receptor is unaffected by bilirubin.

A decrease in the mucosal-to-serosal flux of urea could conceivably lower medullary interstitial osmolality, resulting in impaired countercurrent tubular transport of water and possibly of sodium. In the present studies, bilirubin in the serosal medium appeared to have no effect on basal urea flux. Bilirubin induced a small, but statistically insignificant decrease in urea permeability after stimulation with vasopressin. The major barrier, however, to urea transport lies in the apical membrane of the epithelial cell [10]. Since bilirubin has been shown to bind to biologic membranes and possibly alter their function, bilirubin was placed in the mucosal bathing solution and mucosal-to-serosal urea flux was measured. Under these circumstances, bilirubin again produced no significant effect on urea permeability, either in the basal or vasopressin-stimulated state. Lastly, bilirubin in the serosal bath had no effect on serosal to mucosal flux of urea in the vasopressin (40 mU/ml) stimulated state. Bilirubin did not influence urea fluxes induced by a lower concentration of vasopressin (1 mU/ml). In contrast to the studies of Carvounis et al [20], tissue stimulation with vasopressin, 1 mU/ml, was associated with near maximal urea fluxes and water movement. No separation in the transport of these two substances could be demonstrated with or without bilirubin at this lower concentration of vasopressin. Taken together, these data indicate that the increase in urea permeability induced by vasopressin is not affected by short-term exposure to bilirubin. The possibility that prolonged exposure to bilirubin may have an inhibitory effect on urea transport, cannot be excluded from the present data. Such a possibility may, in fact, account for the decrease in renal medullary urea concentrations found in the Gunn strain of rat.

Short-term exposure to bilirubin produces a limited inhibitory effect on vasopressin-stimulated active sodium transport but does not influence the membrane permeability changes allowing for passive urea and water transepithelial movement. These findings may help explain the apparently conflicting observations in the hyperbilirubinemic newborn infant. In the newborn, alterations in renal function were only observed under conditions where the infant was challenged with a volume and salt load. Since the infants were hyperbilirubinemic for very limited periods of time, the picture of bilirubin nephropathy, as described in the hyperbilirubinemic Gunn rat, never fully developed.

Acknowledgments

This material was presented in part at the Society of Pediatric Research meeting, Washington, D.C., April 1982. The authors thank Mrs. C. McGloin for secretarial assistance. NMR spectra were obtained using a spectrometer purchased with funds from Montedison S.p.A. and the National Science Foundation.

Reprint requests to Dr. A. S. Brem, Division of Pediatric Nephrology, Rhode Island Hospital, 593 Eddy Street, Providence, Rhode Island 02902, USA

References

1. Odell GB, Natzschka JC, Storey GNB: Bilirubin nephropathy in the Gunn strain of rat. *Am J Physiol* 212:931–938, 1967
2. Martinez-Maldonado M, Suki WN, Schenker S: Nature of the urinary concentrating defect in the Gunn strain of rat. *Am J Physiol* 216:1386–1391, 1969
3. Call NB, Tisher CC: The urinary concentrating defect in the Gunn strain of rat. *J Clin Invest* 55:319–329, 1975
4. Whitington PF, Olsen WA, Odell GB: The effect of bilirubin on the function of hamster small intestine. *Pediatr Res* 15:1009–1014, 1981
5. Broberger U, Aperia A: Renal function in infants with hyperbilirubinemia. *Acta Paediatr Scand* 68:75–79, 1979
6. Engle WD, Arant BS: Neonatal hyperbilirubinemia and renal function. *J Pediatr* 100:113–116, 1982
7. Leaf A: On the functional structure of the transport system in the toad bladder, in *Proc 3rd Int Congr Nephrol*, edited by Schreiner G, Basel, New York, Karger, 1967, vol 3, pp 18–26
8. Ussing HH, Zerahn K: Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol Scand* 23:110–127, 1951
9. Bentley PJ: The effects of neurohypophyseal extracts on water

transfer across the wall of the isolated urinary bladder of the toad Bufo marinus. *J Endocrinol* 17:201–209, 1958
10. MAFFLY RH, HAYS RM, LAMDIN E, LEAF A: The effect of neurohypophyseal hormones on the permeability of the toad bladder to urea. *J Clin Invest* 36:630–641, 1960
11. BOND M, SHPORER M, PETERSON K, CIVAN MM: ^{31}P nuclear magnetic resonance analysis of toad urinary bladder. *Mol Physiol* 1:243–263, 1981
12. MACKNIGHT ADC, DIBONA DR, LEAF A: Sodium transport across the toad urinary bladder: A model "tight" epithelium. *Physiol Rev* 60:615–715, 1980
13. LICHTENSTEIN NS, LEAF A: Effect of amphotericin B on the permeability of the toad bladder. *J Clin Invest* 44:1328–1342, 1965
14. JAMISON RL, MAFFLY RH: The urinary concentrating mechanism. *N Engl J Med* 295:1059–1067, 1976
15. HAYS R: Antidiuretic hormone. *N Engl J Med* 295:659–665, 1976
16. SHEPHERD RE, MORENO FJ, CASHORE WJ, FAIN JN: Effects of bilirubin on fat cell metabolism and lipolysis. *Am J Physiol* 237:E504–E508, 1979
17. MORPHIS L, CONSTANTOPOULOS A, MATSANIOTIS N, PAPAPHILIS A: Bilirubin-induced modulation of cerebral protein phosphorylation in neonate rabbits in vivo. *Science* 218:156–158, 1982
18. KAWAI K, COWGER ML: Effect of bilirubin on ATPase activity of human erythrocyte membranes. *Res Commun Chem Pathol Pharmacol* 32:123–135, 1981
19. KASHIWAMATA S, ASAI M, SEMBA RK: Effect of bilirubin on the Arrhenius plots for Na-K-ATPase activities of young and adult rat cerebra. *J Neurochem* 36:826–829, 1981
20. CARVOUNIS CP, FRANKI N, LEVINE SD, HAYS RM: Membrane pathways for water and solutes in the toad bladder. I. Independent activation of water and urea transport. *J Membr Biol* 49:253–268, 1979